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Combinatorial reshaping of a lipase structure for thermostability: Additive role of surface stabilizing single point mutations

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ABSTRACT

Thermostable lipases are of high priority for industrial applications. In the present study, targeted improvement of the thermostability of a lipase from metagenomic origin was examined by using a combinatorial protein engineering approach exploring additive effects of single amino acid substitutions. A variant (LipR5) was generated after combination of two thermostabilizing mutations (R214C & N355K). Thermostability of the variant enzyme was analyzed by half-life measurement and circular dichroism (CD). To assess whether catalytic properties were affected by mutation, the optimal reaction conditions were determined. The protein LipR5, displayed optimum activity at 50 °C and pH 8.0. It showed two fold enhancement in thermostability (at 60 °C) as compared to LipR3 (R214C) and nearly 168 fold enhancement as compared to parent enzyme (LipR1). Circular dichroism and fluorescence study suggest that the protein structure had become more rigid and stable to denaturation. Study of 3D model suggested that Lys355 was involved in formation of a Hydrogen bond with OE1 of Glu284. Lys355 was also making salt bridge with OE2 of Glu284.

1. Introduction

Protein stability is often a limiting factor in the development of commercial proteins and biopharmaceuticals, as well as for biochemical and structural studies [1]. Unfortunately, identifying stabilizing mutations is not trivial since most are neutral or deleterious. Biocatalysts like lipases often need to be robust to meet the harsh reaction conditions [2]. They can catalyze reactions of insoluble substrates at the lipid–water interface, preserving their catalytic activity in organic solvents [3]. This makes lipases the most powerful tool for catalyzing not only hydrolysis, but also various reverse reactions such as esterification, transesterification, aminolysis, or thiotransesterifications in anhydrous organic solvents [4–6]. They display important advantages over classical catalysts, as they can catalyze reactions with reduced side products, lowered waste treatment costs, and under mild temperature and pressure conditions [7]. Accordingly, the use of lipases holds a great promise for green and economical process chemistry [8,9].

However, performance of a lipase is not always sufficient for an industrial application [9] and most enzymes have sub-optimal properties for processing conditions [10]. In order to improve enzyme-mediated process efficiency we need to modify the enzymes suitable for a defined industrial process [9,10]. Knowledge based protein design as well directed evolution based approaches have been used to achieve this goal [11–17].

Thermal stability is a major requirement for commercial enzymes, being critical for industrial applications, as thermal denaturation is a common cause of enzyme inactivation [18]. In effort to meet the industrial demands, many lipases have been engineered to enhance their thermostability, including *Candida antarctica* lipase B [19,20], *Rhizomucor miehei* lipase [21], or *Bacillus subtilis* lipase [22].

In our lab we are actively engaged in the directed evolution of lipases for the last decade [23–29]. Recently, we had isolated lipases from metagenomic sources namely LipR1 and JkP01 [28,29] and evolved them successfully for thermostability as well as enzyme activity. Among them the proteins LipR3 (R214C) and LipM1 (N355K) contain single point mutations [29]. Since, these proteins were highly identical (96% sequence identity) and LipM1 was more thermostable than LipR3, therefore we decided to incorporate mutation of LipM1 to LipR3. We were interested to see, whether these mutations (N355K and R214C) will work additively or not. Therefore an attempt was made to mutate this residue in LipR3 by site directed mutagenesis to demonstrate its effect on thermostability. The mutant (LipR5) containing both the mutation was characterized in detail.

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2. Materials and methods

2.1. Reagents/kits/vectors

All the chemicals and reagents used in the study were of good analytical grade, obtained from Merck (Germany)/Sigma Aldrich (USA)/ HiMedia (India). pGEM-T easy vector used for cloning purpose was purchased from the Promega (USA). pQE30-UA, used as expression vector was purchased from the Quiagen (Germany).

2.2. Cloning of LipR5 by site directed mutagenesis

Site directed mutagenesis was carried out by means of QuikChange® XL site-directed mutagenesis kit obtained from stratagene, according to the manufacturer's instructions. Plasmid of LipR3 was used as template with forward and reverse primers for mutagenesis:

Forward primer 5′ TGGGAACGTACAAAGTCGACCATT 3′
Reverse primer 5′ AATGGTCGACTTTGTACGTTCCCA3′

The nicked vector DNA incorporating the desired mutations was then transformed into XL10-Gold®*** ultracompetent cells.

2.3. Nucleotide sequence analysis and intracellular cloning

To confirm the mutation, the plasmids from the few selected clones were sequenced using universal M13 forward and reverse primer. The nucleotide sequencing was done by commercial available service provided by Chromous biotech (India). Upon sequence analysis, the mutation N355K was confirmed at the desired position. The open reading frame (ORF) of selected lipase gene was cloned intracellularily in pQE-30UA expression vector using primers designed for intracellular cloning (Forward primer 5′-GGATCCATGGCATCTCGACGC-3′ and Normal reverse primers), and expressed in *Escherichia coli* M15 cells containing pREP4 plasmid as per manufacturer's instructions.

2.4. Purification of LipR5

All steps for purification of LipR5 enzyme was carried out at 4 °C (unless otherwise stated) according to the protocol of Kumar et al. (2013) [27]. LipR1 and LipR3 enzymes were also purified simultaneously for comparison.

2.5. Enzyme assay

All the enzymatic assays for determination of residual and relative enzyme activities were carried out according to the method of Kumar et al. (2013) [27]. The total enzyme activity was expressed in U and specific activity was expressed as U/mg of protein. One unit of enzyme activity is defined as the amount of enzyme, which librates 1 μ mol of pNP from pNP-laurate per minute under standard assay conditions. The protein concentration was determined at each purification step using the commercially available BCA (Bicinchoninic acid) kit (Banglore-Genei, India). Bovine serum albumin was used as standard and absorbance was recorded at 562 nm.

2.6. Biochemical characterization

2.6.1. Polyacrylamide gel electrophoresis

The dialyzed protein was analyzed for purity under denaturing condition on 12% SDS–PAGE gel.

2.6.2. Effect of temperature on enzyme activity

Optimum temperature for the LipR5 lipase was determined by assaying the enzyme activity at different temperature (20–80 °C).

2.6.3. Thermal inactivation of enzyme

Thermal denaturation profile of the enzyme was studied by pre-incubating the enzymes separately, at 55 °C, 60 °C, 65 °C and 70 °C respectively. Enzyme aliquots were taken out at different time intervals, cooled at 4 °C for 15 min followed by enzyme assay. Enzyme without incubation was taken as control (100%). Further, the enzyme activity at the start of the experiment was taken as 100%, and the residual lipase activity after incubation was determined. Reaction mix without enzyme served as blank.

2.6.4. Effect of pH on enzyme activity and stability

Optimum pH for the purified lipase (LipR5) was determined by assaying the enzyme in buffers of different pH (sodium acetate—pH 5.0, sodium phosphate—pH 6.0–8.0, Tris–HCl—pH 9.0, Glycine NaOH—pH 10.0–11.0) at 50 °C and 45 °C respectively. The pH stability of the lipase was determined by pre-incubating the enzyme with 0.05 M buffer of different pH (5.0–11.0) for 1 h at room temperature followed by enzyme assay.

2.6.5. Substrate specificity

Substrates specificity for LipR5 was studied using pNP ester (final concentration 0.2 mM) of following chain length: pNP-acetate (C3), pNP-caprylate (C8), pNP-decanoate (C10), pNP-laurate (C12), pNP-myristate (C14), pNP-palmitate (C16), pNP-stearate (C18) from Sigma (USA) were dissolved in absolute alcohol, and assayed according to standard assay method.

2.6.6. Effect of additives and detergents

Effect of various concentrations (10% v/v) of organic solvents, n-Hexane, Acetone, Toluene, Ethylene glycol, DMSO, Glycerol and Methanol, on enzyme activity of both the enzymes were monitored. The purified enzymes (0.1 ml) were incubated with the solvents (10% v/v organic solvent + Sod. Phosphate buffer) for 1 h at room temperature then residual activity was checked by normal enzyme assay protocol. Effect of different additives (0.1 mM each) such as, diethylpyrocarbonate (DEPC), β-mercaptoethanol (β-ME), Tween 20–80 (1%, v/v), Triton X-100 (1%, v/v), sodium dodecyl sulfate (SDS) (1%, w/v), were studied on enzyme activity. The enzymes were incubated with additives at 37 °C for 5 min before enzyme assay. The reaction mix with respective additives but without enzyme served as blank. The reaction mix without any additives was taken as control (100%).

2.6.7. Inhibition study

We have tested the effect of PMSF (a serine inhibitor) on enzyme activity. PMSF (100 μl), of different concentration was added to the reaction mix (Sodium Phosphate buffer 700 μl + 100 μl enzyme). The reaction mix was incubated, at 50, 55 and 60 °C for 3 min respectively, before the substrate (100 μl) was added. The enzyme assays were performed according to standard assays method.

2.6.8. Kinetic parameter

Enzyme activity as function of substrate concentration (0.01–2 mM) was determined for both enzymes. The Michaelis–Menten constant (K_m) and maximum velocity for the reaction (V_{max}) with pNP-laurate as substrate, were calculated by Lineweaver–Burk plot. The k_{cat} and k_{cat}/K_m were also calculated for both LipR3 and lipR5, and the results were compared with LipR1.

2.7. Biophysical characterization

Effect of temperature on enzyme conformation (secondary and tertiary) was studied by CD and fluorescence spectroscopic techniques respectively. Proteins solution from both LipR3 and LipR5 were exposed to temperatures ranging from 20 to 60 °C. Circular dichroism measurements were made with a JASCO J-715 spectropolarimeter fitted with a Jasco Peltier-type temperature controller (PTC-348WI). Instrument was calibrated with D-10 camphorsulfonic acid. The temperature of the protein solution was controlled employing cell holder attached to a Neslab's RTE-110 water bath, with an accuracy of ±0.1 °C. Far-UV CD spectra were taken in the wavelength range of 200–250 nm, at a protein concentration of 15 μM with a 2 mm path length cell. Spectra were collected with a scan speed of 20 nm/min and with a response time of 1 s. Each spectrum was the average of 8 scans. Fluorescence measurements were carried out on a Shimadzu spectrofluorometer (model RF-540) equipped with a data recorder DR-3. The fluorescence spectra were measured at a protein concentration of 10 μM with a 1-cm path length cuvette. To determine intrinsic tryptophan fluorescence, the excitation wavelength was set at 295 nm and emission spectra was recorded in the range of 300–400 nm or at a fixed wavelength of 338 nm with 5 and 10 nm slit width for excitation and emission respectively.

2.8. Molecular modeling

Since these lipases showed high sequence identity (96%) with B. Stereothermophilus P1 (BSP) lipase, whose crystal structure is available, it was possible to develop homology models of the proteins with reliability using Swiss Model. The models were validated by means of PROCHECK and VERIFY3D available at http://nihserver. mbi.ucla.edu/SAVES/.

The nucleotide sequence for *LipR5* have been submitted to GeneBank with accession No. GenBank: KC770105.

3. Results and discussion

3.1. Cloning of LipR5 by site directed mutagenesis

In our efforts to generate highly thermostable lipase LipR5, LipR3 lipase gene was used as a template for site directed mutagenesis. After PCR amplification the amplified products were purified and used to transform XL10-Gold® ultracompetent cells. Sequence analysis of plasmids isolated from selected colonies confirmed a change at position 355 from N → K (SD-Fig. 1).

3.2. Expression and purification of LipR5 enzyme

LipR5 gene (containing the desired mutation) was cloned intracellularly in pQE30-UA vector for expression of protein. The protein was purified, as described in Section 2.6. LipR1 and LipR3 enzymes were purified simultaneously along with LipR5 for comparison (Table 1). The enzymes were purified with good yield and the specific activity of purified LipR5 enzyme (5330 U/mg) was comparable to LipR3 enzyme (6381) (Table 1). The approximate molecular weight of the mutant protein was found to be 44.0 kDa (with His-tag) and the protein migrated at the same position as a single band in 12% SDS–PAGE (SD-Fig. 2).

3.3. Biochemical characterization of LipR5 and comparison with LipR1 and LipR3

3.3.1. Effect of pH on enzyme activity and stability

The enzyme LipR5 was found to be active in wide range of pH from pH 6.0 to pH 10.0. The optimum pH, for all the enzymes were same (8.0) with some change in pH stability. LipR5 was stable in the pH range of 7.0–10.0. It was more stable than LipR3 and LipR1-with more than 80% enzyme activity at pH 7.0–9.0 (Fig. 1A and B). LipR5 showed more than 50% residual enzyme activity at pH 10.0 and ~45% enzyme activity at pH 6.0. It's stability was comparable with that of LipR3 and LipR1 (Fig. 1B).

Table 1
Purification table of mutant (LIPR5).

Purification step	Total protein (mg)	Total enzyme activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Cell lysate					
LipR1	48	875	18	100	1
LipR3	51	4375	86	100	1
LipR5	46	4495	98	100	1
Ni–NTA purified					
LipR1	0.7	547	781	62	43
LipR3	0.6	3829	6381	87	74
LipR5	0.7	3731	5330	83	54

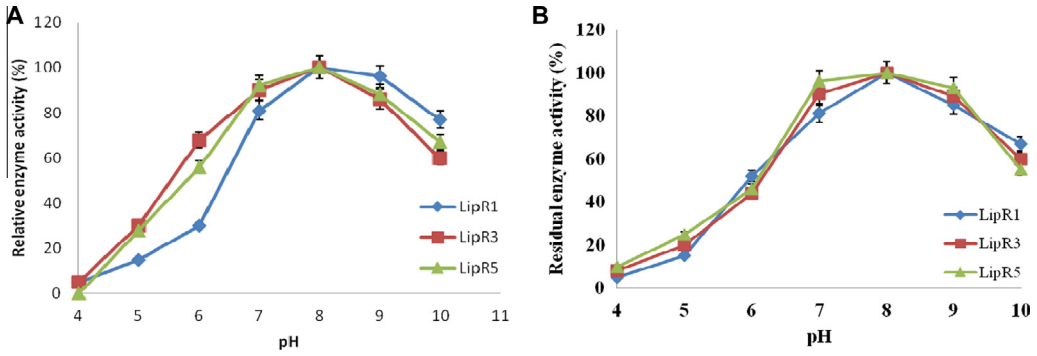

Fig. 1. Effect of pH on the activity (A) and stability (B) of LipR1 (◆), LipR3 (■) and LipR5 (▲) enzymes.

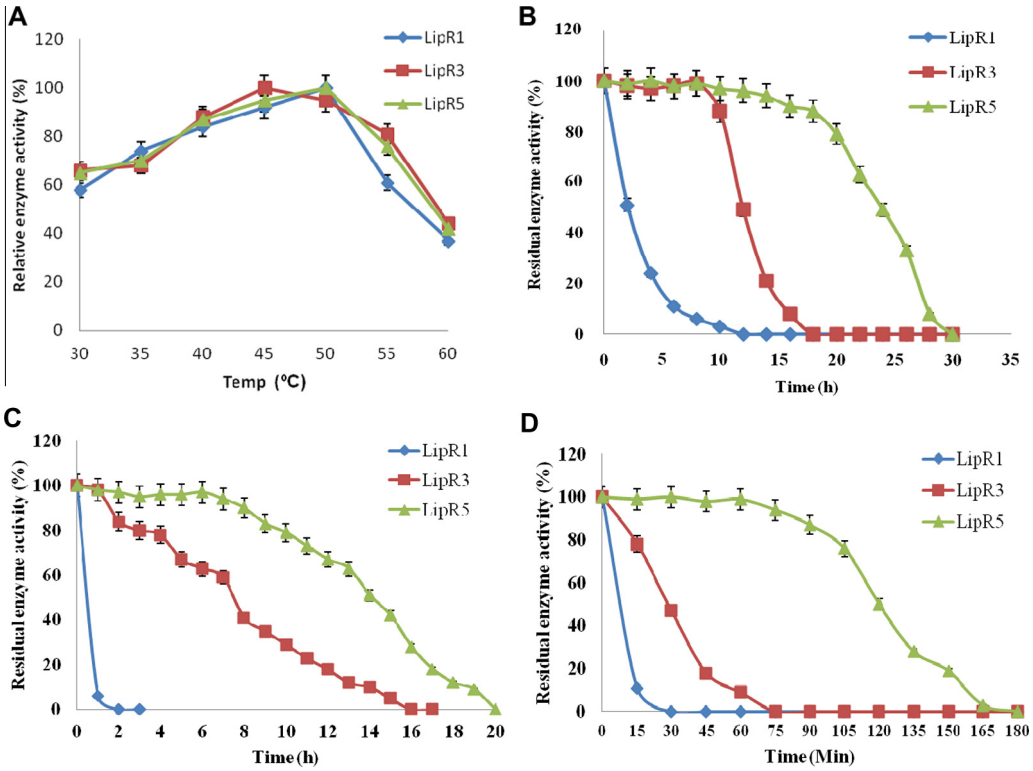

Fig. 2. Effects of temperature on enzyme activities of LipR1 (◆), LipR3 (■) and LipR5 (▲) enzymes (A). Effect of Temperature on stability of LipR1 (◆), LipR3 (■) and LipR5 (▲) enzymes at 55 °C (B), 60 °C (C) and at 65 °C (D).

3.3.2. Effect of temperature on enzyme activity and stability

LipR5 showed optimum activity at 50 °C that is similar to LipR1 but 5 °C higher than lipR3 (45 °C) (Fig. 2A). Thermostability studies of LipR5 was carried out at different temperatures as mentioned in methods and compared with LipR1 and LipR3. LipR5 was quite stable in the temperature range of 50–65 °C. The half-life at 55 °C of LipR5 was found to be ~24 h, much greater than LipR1 (2 h) and LipR3 (16 h) (Fig. 2B). At 60 °C LipR5 showed 168 folds enhancement in half life (14 h) as compared with LipR1 protein (5 min) (Fig. 2C) and ~two fold enhancement in comparison with LipR3 ($T_{1/2}$ 7.5 h). At 65 °C, LipR5 displayed half life of 2 h whereas; LipR3 displayed half life of only 32 min. LipR1 was not comparable as it lost activity instantaneously (Fig. 2D). Its half life above 50 °C was more than both LipM1 and LipR3 (Table 4), Suggestive of additive effect of both these point mutations.

Table 2
Effect of different additives on enzyme activity of LipR5 and LipR3.

	Relative activity (%)			
	LipR3		LipR5	
Control	100 ± 2		100 ± 3	
Inhibitors (1 mM)				
DEPC	12 ± 1		19 ± 1	
β ME	98 ± 5		99 ± 3	
Eserine	97 ± 3		96 ± 1	
PMSF	(1 mM)	(10 mM)	(1 mM)	(10 mM)
At 50 °C	89 ± 2	78 ± 4	99 ± 1	88 ± 2
At 55 °C	58 ± 5	48 ± 1	102 ± 4	84 ± 4
At 60 °C	5 ± 2	7 ± 3	6 ± 0.5	2 ± 0.5
	Organic solvents (10%, v/v)			
n-Hexane	60 ± 3		64 ± 1	
Acetone	55 ± 4		72 ± 3	
Ethylene glycol	65 ± 1		64 ± 1	
DMSO	78 ± 3		103 ± 2	
Glycerol	72 ± 2		87 ± 4	
Methanol	74 ± 4		81 ± 5	

Table 3
Kinetic parameters of LipR1, LipR3 and LipR5 lipases with pNP-laurate as substrate.

Kinetic parameters	LipR1	LipR3	LipR5
K_m (μM)	0.78 ± 0.02	0.63 ± 0.03	0.58 ± 0.03
V_{max} (μmol min^{-1} ml^{-1})	502 ± 7	891 ± 11	712 ± 9
k_{cat} (min^{-1})	977 ± 11	8212 ± 17	7682 ± 19
k_{cat}/K_m (μM^{-1} min^{-1})	1253 ± 13	13034 ± 61	12134 ± 56

Table 4
Comparison of biochemical properties of LipR5 with other lipases.

Biochemical parameters	LipR1(WT)	LipR3	LipM1	LipR5
Optimum pH	8.0	8.0	8.0	8.0
pH stability	7.0–10.0	7.0–10.0	8.0–11.0	7.0–11.0
Optimum Temp.	50 °C	45 °C	40 °C	50 °C
Substrate specificity	C-12	C-12	C-16	C-12
Half life at 50 °C	2 h	12 h	20 h	24 h
Half life at 60 °C	5 min	7.5 h	12 h	16 h
Half life at 65 °C	~5 min	32 min	75 min	2 h
References	[27]	[26]	[28]	Current study

3.3.3. Effect of additives and inhibitors

The enzyme LipR5 was found resistant to all the organic solvents tested (Table 2). LipR5 was found to be more resistant to triton X-100, tween-60 and tween-80 as compared with LipR3 and LipR1 (SD-Fig. 3A). PMSF showed temperature dependent inhibition of enzyme activity with all the proteins LipR1, LipR3 and LipR5 (Table 2). LipR5 showed resistance to inhibition till 55 °C, but at 60 °C, it's activity was inhibited completely, suggestive of a tem-

perature switch for the complete opening of catalytic site. Both, LipR1 and LipR5 showed strong inhibition in the enzyme activity with 1 mM DEPC. Metal ions had not much effect on enzyme activities of enzymes (SD-Fig. 3B).

3.3.4. Substrate specificity and Kinetic analysis

Optimal substrate for the enzyme was found to be pNP-laurate. Substrate preference of LipR5 protein was in the order of C-12 > C-10 > C-8 > C-16 > C-14 > C-18 > C-4 > C-2 (Table 2). Enzyme activity of the lipases as a function of substrate concentration (0.05–2 mM) was carried out. The kinetic parameters such as, K_m, V_{max}, k_{cat} and k_{cat}/K_m of LipR5 and other proteins demonstrated great variation. There was decrease in the K_m value for LipR5 (0.58) as compared to LipR1 (0.78). There was increase in catalytic efficiency of LipR5 as compared to LipR1 (Table 3). However kinetic parameters of LipR5 were found to be similar to LipR3.

3.4. CD and fluorescence studies

Far-UV CD spectra for LipR5 displays strong negative bands in the region of 200–250 nm. LipR5, showed minimal loss in secondary structure on increasing temperature from 20 °C to 60 °C (Fig. 3B). Thus LipR5 structure is more robust as compared to LipR1 and LipR3 (Fig. 3A). Investigation of structural changes using intrinsic tryptophan fluorescence as a function of temperature, (Fig. 3C–E), it was observed that LipR5 displayed biphasic unfolding behavior. In case of LipR1, the emission maxima drastically decreased after 30 °C indicating significant unfolding, while in case of LipR5 little change in emission maxima was observed even up to 55 °C. This clearly indicated that LipR5 is more thermostable as compared to LipR3 and LipR1. Increase in surface hydrophobicity can be correlated from the studies with ANS binding (Fig. 3F). A sharp increase in fluorescence intensity of LipR5 as compared to

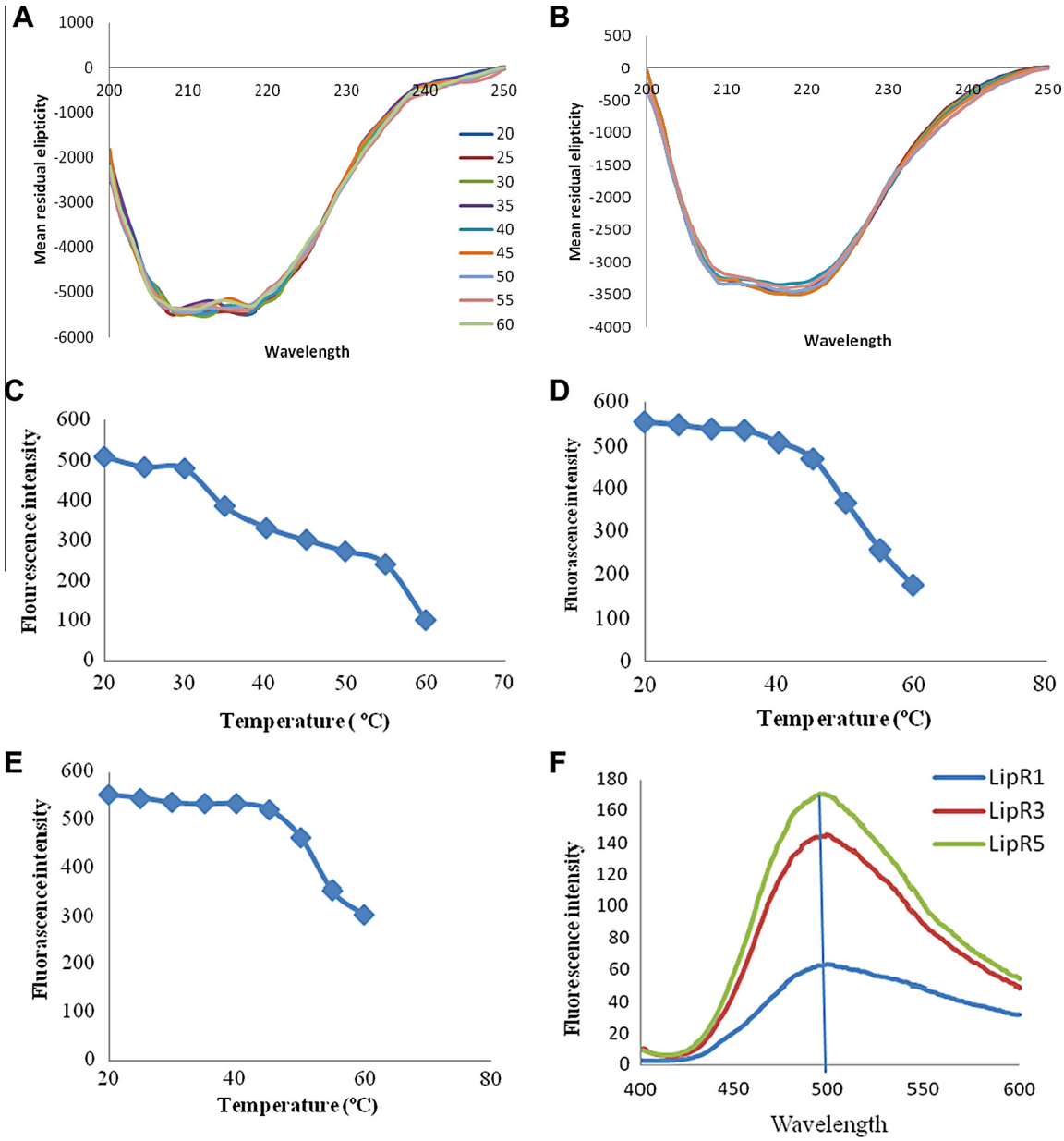

Fig. 3. Far UV-CD of lipase recorded in the 250–200 nm range from 20 to 60 °C for LipR3 enzyme (A) and LipR5 enzyme (B). Effect of temperature on intrinsic tryptophan fluorescence of LipR1 (C), LipR3 (D) and LipR5 (E) recorded at 338 nm. Fluorescence spectra of LipR5, LipR3 and LipR1 in the presence of bis-ANS (4,4′-Dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt) (F). A line is drawn over the emission maximum of bis-ANS.

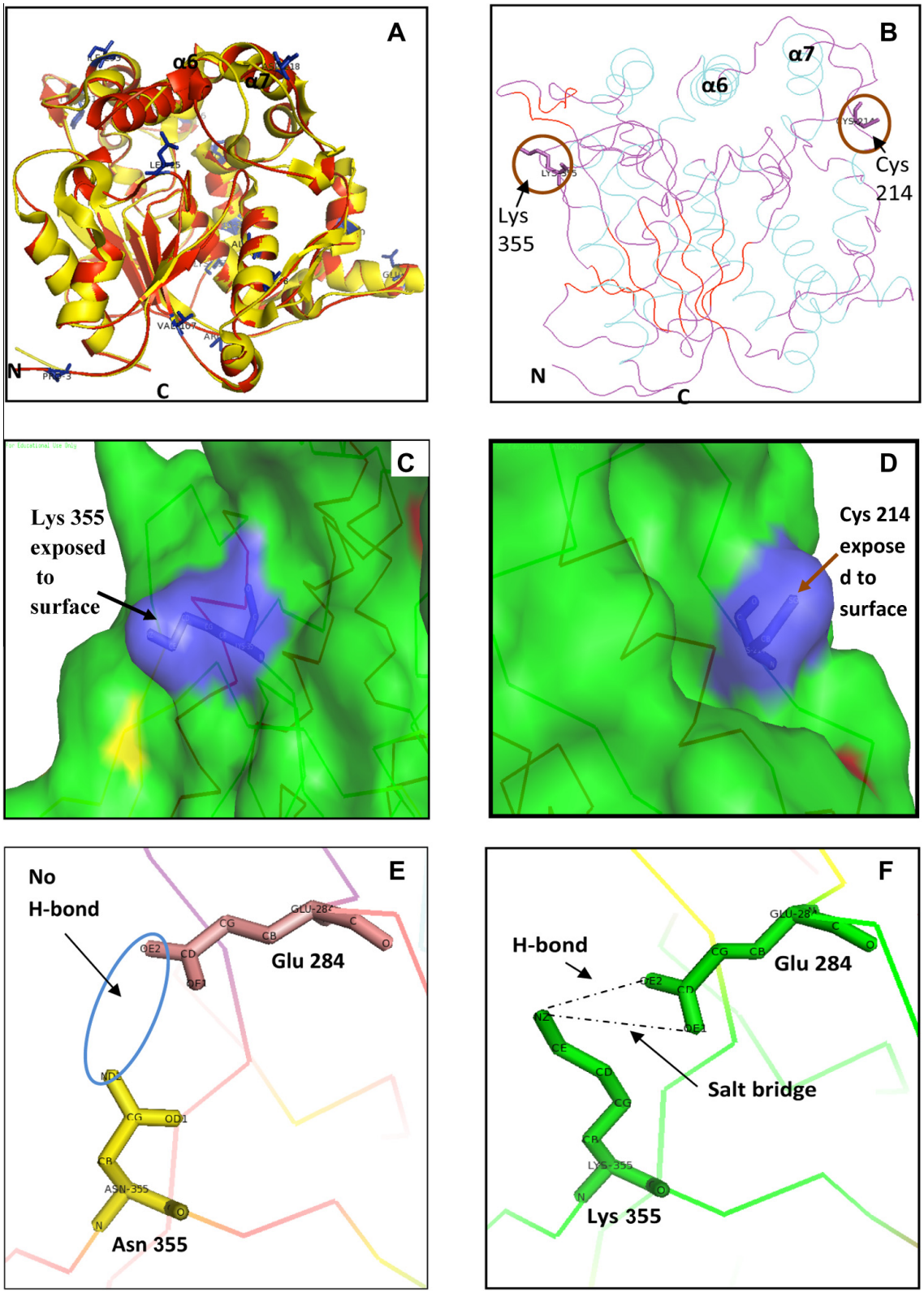

Fig. 4. Superimposition of Cα backbone of wild type model (LipR1) with LipR5 (A) residues in blue color showing, differences with BSP. Ribbon diagram showing LipR5 and positions of changed amino acids (blue) (B). Surface representation of Cα superimposition of model of LipR5 illustrating the positions of the substituted amino acids N355K & R214C (C & D). In the LipR1 model, Asn355 and Glu284 were far apart (E), but in LipR5 the side chain of Lys 355 came near to Glu284 (F) and formed H-bond with distance of 2.9 Å as well as salt bridge with distance of 2.9 Å. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

LipR3 and LipR1 demonstrated that the surface hydrophobicity of LipR5 had been increased due to addition of hydrophobic residue (Cys) at the surface.

3.5. Homology modeling and analysis of possible interactions, responsible for thermostability of LipR5

Upon visualization of LipR5 model (Fig. 4A and B), the mutation R214C was found to be in the loop connecting α6 and α7 helices, which is exposed to solvent and thus is on the protein surface (Fig. 4B and D). Second mutation N355K was also on protein surface (Fig. 4C).

The mutation N355K has also been reported in other thermostable lipases [30]. It has been well reported that substitution of asparagine to lysine often lead to enhanced thermostability by either making Hydrogen bonds or salt bridges [31,32]. There are reports which states that Asn at surface get readily aminated and thus decreases the thermostability of protein [32]. Therefore, replacement of Asn with Lys in LipR5 prevented the enzyme from possible amination and thus provided rigidity to the protein. It's high thermostability can also be attributed to the involvement of the residue Lys355 in H-bond formation with oxygen (OE1) of Glu284 (Fig. 4E and F) as well as salt bridge with OE2 of Glu284 (Fig. 4F) with distances of 2.9 and 3.1 Å respectively. Since the mutation N355K was close to an active site residue His358 (SD-Fig. 1), therefore, it's addition had enhanced the hydrophobic interactions in the protein core with other active site residues. This might have provided more rigidity to LipR5 structure. This might also play role in maintaining the catalytic efficiency of the protein. Thus addition of second mutation (N355K) in LipR3 worked additively and enhanced the thermostability of the protein without compromising with other properties

In Summary, we have successfully developed a highly thermostable lipase with the combination of two surface stabilizing single point mutations by site directed mutagenesis without compromising the enzyme activity. This study will help to understand the additive effect of surface stabilizing mutations.

Conflict of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.051.

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